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Generation and Assembly of Human Brain Region-Specific Three-Dimensional Cultures

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Abstract

The ability to generate region-specific 3D models to study human brain development offers great promise for understanding the nervous system in both healthy individuals and patients. In this protocol we describe how to generate and assemble subdomain-specific forebrain spheroids, also known as brain-region specific organoids, from human pluripotent stem cells (hPSCs). We describe how to pattern the neural spheroids towards either a dorsal forebrain or ventral forebrain fate, establishing human cortical spheroids (hCS) or, human subpallial spheroids (hSS), respectively. We also describe how to combine the neural spheroids in vitro to assemble forebrain assembloids that recapitulate the interactions of glutamatergic and GABAergic neurons seen in vivo. Astrocytes are also present in the human forebrain-specific spheroids and these undergo maturation when the forebrain spheroids are cultured long-term. The initial generation of neural spheroids from hPSCs occurs in less than one week, with regional patterning occurring over the subsequent 5 weeks. After the initial maturation stage, brain region-specific spheroids are amenable to a variety of assays, including live cell imaging, calcium signaling, electrophysiology,

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AUTHOR CONTRIBUTIONS

S.A.S., J.A., AM.P., F.B. collected data and contributed to the optimizations of the protocols. S.A.S. and S.P.P. wrote the manuscript with input from all authors. S.P.P. supervised this work.

CONFLICT OF INTEREST

The author declares no competing financial interests.

Anticipated Results

Successful differentiations should yield about 20–40 mature spheroids per 10-cm plate of hPSCs. When hPSC colonies are ready for formation of spheroids, they should not be touching each other and have no evidence of central differentiation (Figure 2a, Figure 3a). Following dispase treatment, detachment of hPSC colonies should occur in under 45 minutes (Figure 3b). Floating spheroids will be visible by eye. Throughout neural differentiation and maturation, spheroids should grow in size up to 4 mm in diameter (Figure 2c; see also Pasca, Sloan et al.[17]), and should be uniform in distribution across the same hPSC line. They should not be fused or touching each other in the ultra-low attachment plate (Figure 3c, d). At early stages, hCS should express the forebrain markers FOXG1 and PAX6 (e.g., day 25) and then exhibit proliferative zones with ventricular-like and subventricular-like domains that are surrounded by a cortical plate-like region (Figure 4a, b, d). hSS should express FOXG1 and the ventral forebrain maker NKX2.1. By day 60, hSS should contain GAD67 and SST immunopositive neurons (Figure 4a, c, e). Astrogenesis should start after day 80–90 and continue over the next several months[20]. For forebrain assembloids, migration of GABAergic neurons into hCS should occur 1–3 weeks post-fusion (Figure 4f–g). Migrating interneurons should disperse underneath the surface and throughout the parenchyma of the hCS, and the integrate into the network (Figure 4i). Examples of hCS or hSS experiments using calcium imaging, live imaging and iDISCO can be found in Figure 4 of Pasca, Sloan et al.[17], Figure 2 and Figure 3 of Birey et al.[19], and Figure 2 and Extended Figure 10 of Birey et al.[19], respectively.

cell purification, single cell transcriptomics, and immunohistochemistry. Once generated, forebrain spheroids can also be matured for over 24 months in culture.

INTRODUCTION

Understanding the development of the human nervous system and elucidating the mechanisms that lead to brain disorders represent some of the most challenging ongoing endeavors in neurobiology. One significant obstacle is the restricted access to healthy and diseased human brain tissue for functional molecular and cellular studies[1,2]. As a result, experimental paradigms have been largely confined to animal models or *in vitro* cell culture systems that do not fully recapitulate the developmental, architectural, and species-specific aspects of the human brain. Thus, there is a great need for human-derived model systems that recapitulate features of human central nervous system (CNS) development and allow for the study of these processes in both healthy and disease conditions.

Modeling human neural development with pluripotent stem cells

The ability to reprogram human somatic cells to induced pluripotent stem cells (hiPSCs)[3–5] and subsequently differentiate these cells to neural lineages provides the opportunity to study features of human neural development *in vitro*[6]. Additionally, these systems allow scientists to link rare, highly penetrant mutations as well as more complex genetic events to cellular disease phenotypes[7,8]. Initial patient-derived hiPSCs models centered around the use of adherent two-dimensional (2D) cell culture systems[9–13]. These protocols generate relatively homogeneous populations of neural types, but do not capture the cellular diversity or tridimensional (3D) tissue architecture[14]. 3D human brain cultures were established to provide physiologically relevant models that more faithfully recapitulate the spatial organization, cellular diversity, and cell-cell interactions that are present in the developing human nervous system[15,16].

In this protocol, we describe how to generate brain region-specific spheroids from hiPSC or human embryonic stem cells (hESC). These region-specific spheroids or organoids are patterned to resemble the dorsal forebrain, also known as the pallium (human cortical spheroids, hCS)[17,18], or the ventral forebrain, also known as the subpallium (human subpallial spheroids, hSS)[19]. The protocol comprises neural induction of 3D aggregates of human pluripotent stem cells (hPSCs) followed by culture in the presence of a variety of small molecules to derive specific cell fates. We have previously used this protocol for the *in vitro* study of the development of the human cerebral cortex[17] and the maturation of glial cells into postnatal stages[20], to test infectivity in human neural cells of novel AAV subtypes[21], to model inter-regional cross-talk in the developing forebrain and to identify phenotypes in a monogenic form of autism spectrum disorders. Because neural spheroids are derived from reprogrammed human cells, they are an ideal system for studying disease phenotypes from specific human genetic backgrounds[18]. Additionally, this platform allows for the study of later stages of human brain development, including the maturation of astrocytes[20], and can be used to derive and assemble different region-specific spheroids into *brain assembloids*, which can be used to study cell migration and neural circuit formation[19].

Features of human forebrain development

Human forebrain development proceeds through a series of stereotyped steps that are guided by specific patterning cues[22,23]. Early in embryonic development, the neural tube begins to expand and forms three primary pouches, which are anatomic structures that will eventually segregate into forebrain (prosencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon)[24]. The maturing forebrain can be anatomically and functionally subdivided along a dorsal–ventral axis, with the dorsal region (pallium) eventually generating the bulk of the cerebral cortex and the ventral region (subpallium) comprising the lateral and medial/caudal divisions of the ganglionic eminences (LGE, MGE/CGE, respectively)[25]. These eminences are the origin of cortical interneuron populations[26]. The vast majority of GABAergic interneurons are born in the MGE or the CGE, where they also acquire specific subtype identities (e.g., parvalbumin, somatostatin, calbindin, etc.). Having been specified in the subpallium, these interneurons begin a migratory journey to populate the developing dorsal pallium. Around gestational week (GW) 15 in humans, interneurons exit the pallial-subpallial border and enter the dorsal forebrain via tangential migration to disperse throughout the subplate, cortical plate, and marginal zones[27]. This process continues in frontal cortical regions from then onward and throughout the second year of life in humans[28]. These cortical interneurons then undergo activity-dependent maturation and integration into neural circuits[29] where they contribute to the excitation to inhibition balance (E/I). This is a critical process in CNS development and circuit functioning. Furthermore, genetic or environmental imbalances of the E/I interplay are thought to contribute to neuropsychiatric disorders, such as epilepsy, autism spectrum disorders, and intellectual disabilities[30,31].

Neuronal production is just one of the many components of CNS development. During later gestational stages and continuing after birth in humans, neural progenitors switch from a largely neurogenic to a gliogenic fate, generating astrocytes and later oligodendrocytes[32,33]. This transition constitutes the bulk of astrogenesis, as newly born astrocyte precursor cells begin to populate the brain parenchyma and distribute throughout the CNS in a tiled distribution. Astrocytes help choreograph neural development and actively contribute to neural circuit formation by controlling synapse formation, function, and elimination. This raises the possibility that astrocyte dysfunction may also directly contribute to the pathophysiology of neuropsychiatric disorders.

Development of the method and comparison to other approaches

In recent years, a number of 3D brain tissue models have been developed[16,17,34–37], each with specific advantages and disadvantages. What is common among these methodologies is that they utilize the capacity of hPSC to differentiate and self-organize in a 3D environment and to recapitulate key features of human brain development, such as the presence of ventricular-like structures surrounded by progenitor populations that differentiate into multiple cell lineages.

The method we describe here builds upon several previous advances. Inhibitors of the SMAD pathway were shown to induce rapid and fast neural differentiation of high density hPSCs with a default dorsal forebrain fate[38]. In addition, hPSCs were shown to undergo

neural differentiation following aggregation in the presence of a ROCK inhibitor in V- or U-shaped wells followed by plating in 2D cultures several weeks later[39]. Building upon these studies, we developed an approach that involved transferring intact colonies of hPSCs to ultra-low attachment plates, and culturing these cells exclusively in suspension without an extracellular matrix or a bioreactor in the presence of two inhibitors of the SMAD pathway to facilitate neuralization [17]. For neuralization, we have primarily used dorsomorphin (DM) and SB-4321542 (SB), but other combinations of SMAD inhibitors can be used. Our approach also uses a longer exposure to the growth factors EGF and FGF2 to allow proliferation and corticogenesis progression, followed by an 18-day exposure to BDNF and NT3 for maturation. This protocol yields self-organizing 3D cultures resembling the dorsal forebrain, which can be maintained for years displaying cellular features observed in the postnatal brain[20]. We have subsequently developed a complementary approach to derive ventral forebrain 3D cultures in which double SMAD inhibition is followed by exposure to a small molecule sonic hedgehog (SHH) agonist, retinoic acid for fate specification, and Allopregnanolone (AlloP) for neural maturation[19].

Overall, our method yields region specific 3D forebrain cultures. In the years following the development of the protocol outlined here, a number of additional groups have continued to adapt the strategy of regional patterning to obtain other brain region-specific patterned structures, such as midbrain, hindbrain or cerebellum[17,35–37,40]. In addition, and in contrast, other groups have combined the intrinsic differentiation ability and the inherent self-differentiation cues within hPSCs with extracellular matrices or bioreactors to develop organoids via an undirected approach[34]. Without specific inductive signals, this approach has the advantage of generating a variety of cell fates within and across organoids. Single-cell transcriptomic studies of undirected organoids[41] confirm the present of mixed dorsal and ventral forebrain cell lineages, along with cells from other brain regions, such as hindbrain, midbrain, retina, and also mesodermal cells. This high degree of cellular diversity may allow for future studies into human CNS diversity and mapping of genes associated with neuropsychiatric disease onto specific cell types[16]. However, given the lack of external cues, these undirected differentiation techniques tend to display a higher degree of stochasticity and may exhibit a degree of variation in cell types and maturation that can make interpreting disease phenotypes challenging.

One application of the region-specific brain spheroids is for modeling inter-regional interactions in CNS development[19]. In this modular approach, separately patterned ventral and dorsal forebrain spheroids are cultured together so that they fuse and generate forebrain assembloids. This enables cortical GABAergic neurons, which are specified in ventral forebrain spheroids, to migrate towards the dorsal forebrain spheroid and then to synaptically integrate into networks with cortical glutamatergic neurons. This platform allows live monitoring as well as genetic and pharmacological manipulation of cortical interneuron migration, a process that happens at late stages in human gestation. Importantly, we have compared our findings with results seen from live imaging of human primary fetal forebrain slices (GW20). We have also demonstrated the power of this platform to identify cell-specific phenotypes and rescue strategies by deriving forebrain assembloids from patients with a rare genetic form of autism spectrum disorder and epilepsy[19]. Two other groups have subsequently adapted similar, assembly-based approaches to recreate and

modulate cell migration from ventral forebrain (subpallium)-like to dorsal forebrain (pallium)-like 3D cultures [42,43]. Bagley et al. [42] used matrigel-embedding and orbital shaker for assembly, while Xiang et al. [43] implemented a spontaneous fusion approach. Each study also used unique molecular labeling approaches to track migrating cells from one 3D culture to the other. For example, Bagley et al. used an EF1α-GFP expressing hPSC line to track the population of migrating cells (60% of which were GABAergic cells), while Xiang et al. used an NKX2.1-GFP hPSC line to track the MGE-lineage cells, similar to the Dlxi½b::GFP enhancer we have utilized. These studies show that brain assembloids can reproducibly model interregional interactions and elaborate cellular phenotypes such as cell migration in a 3D microphysiological environment.

Applications and limitations

hPSC-derived 3D cultures can be used to address biological questions that are related to human brain development and disease modeling[15,44]. One particular advantage of this method is the ability to investigate human-specific phenotypes in patient-derived cultures, which may be challenging to capture in other systems. The entirety of the protocol described here is performed in ultra-low attachment culture plates without embedding into extracellular matrices or the use of spinning bioreactors. At later stages of differentiation, cell death may be observed in hCSs and hSSs. However, our biosensor measurements demonstrate a partial pressure of oxygen that remains >60 mmHg in the center of hCSs that are 4 mm in diameter.

The brain region-specific spheroids we obtain are amenable to many downstream assays and analyses (Figure 1). They can be fixed and cryosectioned for immunohistochemistry (step 23 option C) or *in situ* hybridization and they can be used intact for live cell imaging using a variety of microscopy setups (for example step 23, options D and F). Additionally, they can easily be dissociated for sorting into distinct cell populations (step 23 option B), immunopanning or single cell transcriptomics. Forebrain spheroids resembling the dorsal or ventral part can also be fused to form assembloids[19,42,43] (step 23, option A). This system captures the interactions of various regional-specific neuronal and/or glial populations, including the migration and integration of GABAergic neurons into the cerebral cortex. Forebrain spheroids can be cryosectioned for immunostaining (step 23 option C) and are amenable to physiology experiments in slices. They can also be loaded with calcium-sensitive dyes for probing neuronal activity (see[19] for detailed protocols). Neural spheroids can easily be transfected at any stage with viruses carrying a variety of constructs (box 1) and can even be transplanted with exogenous cell types such as microglia or oligodendrocytes, which engraft and migrate into the parenchyma of the 3D structure.

The system is easily scalable and therefore can be used for toxicology, CRISPR/Cas9 genome editing and drug screening paradigms. Additionally, while the protocol described here includes growing hPSCs on MEFs, optimization to feeder-free and xeno-free conditions are currently in process and so should be achievable. Perhaps most importantly, forebrain spheroids can be maintained in suspension culture for long periods of time[20] (over 25 months in our experience), which allows for the study of cell diversity and maturation as the spheroids age *in vitro*.

Nevertheless, whilst there has been unprecedented progress in the ability to differentiate hPSCs into cell types of interest and rapid advances in ability to grow cells in 3D cultures, there are still significant issues that need to be addressed. These include the need to promote further functional maturation of neurons and glial cells, which could require more physiological media formulations, improved extracellular matrices or novel strategies for perfusion and grafting into mammalian tissue[16]. To capture the cellular diversity and study complex cell–cell interactions in the CNS, future organoid or assembloid models could incorporate myelinating oligodendrocytes, microglia, and vascular cell types. Variability between organoids is a barrier to disease modeling, and thus tools for controlling their internal organization, such as the use of organizer-like molecules (e.g., morphogens soaked in beads), could become powerful strategies. Developing strategies for accelerating the maturation and senescence of human neural cells will be critical for developing *in vitro* models of neurodegenerative disorders. Lastly, there is a need to be able to scale up culture systems to facilitate running large drug and genetic screens or studies across large cohorts of patients and controls. Over the next years, as the field evolves, this technology promises to accelerate the study of human brain development, evolution and disease.

Experimental design

Prior to beginning experiments, it is important to consider several key points that will help determine the scale and time required to perform adequately powered differentiation experiments:

- All experiments should be performed using at least 3 hPSC lines, with at least 3 separate differentiations, and using at least 2–3 neural spheroids per differentiation or condition.
- If validating disease-phenotypes, the use of isogenic hPSC lines is essential. .
- Results from hESC and hiPSC experiments should be reported separately. We have successfully generated neural spheroids from from over 70 hiPSC lines and hESCs, such as H9.
- Some line-to-line heterogeneity (especially in early stages of differentiation) can be expected, but there should be consistency within lines and across separate differentiations of the same line.
- For intra-spheroid quantifications, at least 3–5 sections per spheroid should be used.

Additionally, it is important to undertake the following quality control steps during the differentiation and patterning process:

- Cultures of hPSC, hCS and hSS need to be regularly checked for Mycoplasma (1–2 times/month)
- Genome integrity of hPSC needs to be verified regularly by SNP array or comparative genomic hybridization arrays (CGH) to identify *de novo* genomic events. If any abnormalities are noted, earlier passages of cells should be used. For differentiations, we primarily use hPSC lines between passages 10 and 40.

- The quality (size, shape, tendency to differentiate) of hPCS needs to be checked daily before starting a new differentiation experiment. It is important to ensure the properties of hPSCs are consistent across passages and before differentiations.
- Cultures should be checked regularly for bacterial/fungal infection. If media is found to be cloudy it should be checked under the microscope at 40× magnification to identify moving bacteria or fungi. It is important to determine the type of contamination immediately and take appropriate measures.
- From day 0 to 6 of differentiation, newly formed neural spheroids need to be checked daily for disintegrating/dying cells (media will be cloudy at the bottom of the plate and lots of floating single cells may be observed under the microscope). If cell death or spheroid disintegration exceeds expectations, it is likely the differentiation will not progress and is better to discard the plates.
- The health of spheroids may vary over the culture period and be dependent on the quality of the founder hPSC colonies. Consider sampling hCS or hSS and carrying out periodic immunostainings to assess health. Generally, autofluorescence in the center of sectioned spheroids or detaching cells on the surface indicates cell death.
- Consider introducing quality controls for differentiation efficiency by running qPCRs for a panel of fate and region-specific genes at particular time points during the differentiation (e.g., day 25).

Overview of Procedure

The procedure starts with culture and passage of hPSCs on a MEF feeder layer (steps 1–13). When hPSC colonies reach around 1.5 mm diameter, they are suspended in media (steps 14–16) and cultured in specific culture conditions that induce neuronal differentiation (steps 17–21). Step 22 describes the specific culture conditions required to differentiate neural spheroids into hSS or hCS. In step 23 of the procedure we describe how to carry out various downstream assays. We also describe how to virally label hCS and hSS, which can be carried out at any stage of spheroid growth, in Box 1.

MATERIALS

Reagents:

Cells

– Human pluripotent stem cells (hPSCs); forebrain spheroids can be generated from cultures of both hESCs e.g. H9, or hiPSCs. ! CAUTION: The experiments involving hPSCs in this study were approved by the Stanford University School of Medicine IRB and Stem Cell Research Oversight (SCRO). The hPSCs used to generate the results shown in this protocol were derived at Stanford University and validated using standardized methods (pluripotency assays, genome integrity, etc), as previously described[10,45,46]. Cultures were tested and maintained mycoplasma-free. All experiments involving the use of hPSCs must

conform to institutional and federal regulations including IRB panel approval and appropriate informed consent.

- EmbryoMax Primary Mouse Embryo Fibroblasts, Neo Resistant, Myto-C treated (Millipore, cat. no PMEF-N-K). Alternatively, DR4 Mouse Embryo Fibroblasts can also be used.

Media and supplements

- DMEM (Life Technologies, cat. no. 10313–039)
- DMEM/F12 (1:1, Life Technologies, cat. no. 11330–032)
- Neurobasal A (Life Technologies, cat. no. 10888022 ! CAUTION: Neurobasal A is extremely sensitive to changes in temperature and light. Keep refrigerated at all times.
- Fetal Bovine Serum, certified, (Life Technologies, cat. no. 16000–044) ! CRITICAL: in order to avoid lot-to-lot variability biases, we recommend to test and purchase this product in bulk.
- KnockOut Serum Replacement (Life Technologies, cat. no. 10828–028) ! CRITICAL: in order to avoid lot-to-lot variability biases, we recommend to test and purchase this product in bulk.
- B27 supplement (Life Technologies, cat. no. 12587010) ! CRITICAL: B27 supplement without vitamin A. In order to avoid lot-to-lot variability biases, we recommend to test and purchase this product in bulk.
- GlutaMax Supplement, 200 mM (Life Technologies, cat. no. 35050–061)
- MEM Non-Essential Amino Acids Solution, 100X (Life Technologies, cat. no. 11140–050)
- Penicillin-Streptomycin (Pen-Strep)*, 10,000 U/ml (Life Technologies, cat. no. 15070–063)

  CRITICAL: Differentiations can also be performed without Pen-Strep
- 2-Mercaptoethanol (Sigma-Aldrich, cat. no. M3148)
- Human-recombinant FGF2 (R&D Systems, cat. no. 233-FB)
- Rock Inhibitor Y-27632 (Selleckchem, cat. no. S1049)
- SMAD inhibitor Dorsomorphin (aka Compound C) (Sigma-Aldrich, cat. no. P5499)
- SMAD inhibitor SB-431542 (R&D Systems/Tocris, cat. no. 1614)
- Human-recombinant EGF (R&D Systems, cat. no. 236-EG)
- Wnt inhibitor, IWP2 (Selleckchem, cat. no. S7085)
- Smoothened Agonist, SAG (Selleckchem, cat. no. S7779)
- Allopregnanolone (Cayman Chemicals, cat. no. 16930)

- Retinoic Acid, RA (Sigma-Aldrich, cat. no. R2625)
- Human-recombinant brain-derived neurotrophic factor, BDNF (PeproTech, cat. no. 450–02)
- Human-recombinant neurotrophin 3, NT3 (PeproTech, cat. no. 450–03)

Cell culture reagents

- Gelatin Solution Bioreagent 2% in H_2O (Sigma, cat. no. G1393)
- Dispase (Invitrogen, cat. no. 17105–041)
- Dulbeco's PBS with calcium and magnesium (Caisson Labs, cat. no. PBL02)
- Dimethyl sulfoxide, DMSO (Sigma-Aldrich, cat. no. D2650)
- Ethyl ethanol 200 proof (Gold Shield, cat. no. 412804) ! CAUTION Ethyl ethanol is flammable and should be maintained in a flame protective cabinet

For single cell dissociation only:

- 1x Earle's balanced salt solution (EBSS, Sigma, cat. no. E7510)
- Bovine serum albumin (Sigma, cat. no. A4161)
- 0.4% DNAse, 12,500 units/ml (Worthington, cat. no. LS002007)
- L-cysteine hydrochloride monochloride (Sigma, cat. no. C7880)
- Papain (Worthington, cat. no. LS 03126)
- Trypsin inhibitor (Worthington, cat. no. LS003086)
- Low Ovo (10x). See Reagent setup.
- High Ovo (10x). See Reagent setup.

For cryosectioning and immunostaining only

- 16% Paraformaldehyde (PFA) (Electron Microscopy Sciences, cat. no. 15710) ! CAUTION PFA must be handled in a safety cabinet and should be disposed of according to institutional guidelines.
- Sucrose (Sigma-Aldrich, cat. no. S9378)
- Fisher Healthcare Tissue-Plus O.C.T Compound– Clear (Fisher Scientific, cat. no. 23–730-571)
- Triton X-100, laboratory grade (Sigma-Aldrich, cat. no. X100)
- Normal donkey serum (EMD Millipore, cat. no. S30-M)
- Primary antibodies (See Table 1)
- Secondary antibodies, species-specific anti IgG (H+L)– Alexa Fluor-conjugated (Molecular Probes and Jackson Immunoresearch)

- Hoechst 33258, Pentahydrate (bis-Benzimide) (Life Technologies, cat. no. H3569)
- Aqua-Poly/Mount (Polysciences, cat. no. 18606)

For optical clearing of intact spheroids by iDISCO only

- Methanol (Certified ACS, Fisher Chemical, cat. no. A412–1)
- Hydrogen peroxide, H_20_2 (Certified ACS, Fisher Chemical, cat. no. H325–100)
- Glycine (Sigma-Aldrich, cat. no. G7126)
- Goat serum, heat inctivated (MP Biomedicals, cat. no. 092939249)
- Heparin sodium salt (Sigma-Aldrich, cat. no. 84020)
- Tween 20 (Sigma-Aldrich, cat. no. P1379)
- Tetrahydrofuran anhydrous (Sigma-Aldrich, cat. no. 186562)
- Dichloromethane (Sigma-Aldrich, cat. no. 270997)
- Benzyl ether (Sigma-Aldrich, ca. no. 108014)
- Tetrahydrofuran (THF)
- Dichloromethane (DBE)
- 4 mL E-C Borosilicate Glass Screw Thread Vials with TFE-Lined Caps (Wheaton, cat. no.: 03–343-6C)
- RS Hughes Inc RTV108 2.8OZ Silicone Sealant (Fisher Scientific, cat. no. NC0380109)
- Rectangle cover glasses (Fisher Scientific, cat. no. 22–266973)

For live imaging of cell migration and calcium signaling only

- Environmental chamber in confocal microscope
- Glass-bottom plates (Corning, cat. no. 4580)

Equipment

- Primaria Cell Culture Dish, 10 cm (BD Falcon, cat. no. 353803)
- Sterile plastic tubes, 15 ml and 50 ml (Corning, cat. nos. 430791 and 430829)
- Ultra-low attachment plates (Corning, cat. nos. 10 cm: 3262; 6 cm: 3261; 6-well: 3471; 24-well: 3473)
- Fisherbrand Premium Microcentrifuge Tubes– Natural: 1.5 ml (Fisher Scientific, cat. no. 05–408-129) ! CAUTION Autoclave tubes before use
- Sterile plastic pipettes (Corning, cat. nos. 5 ml: 356543, 10 ml: 356551, 25 ml: 356525, 50 ml: 356550)

- Sterile tips (MBP ART, cat. nos. P1000: 2779-HR, P200: 2769-HR, P20: 2749-HR)
- Sterilized scissors
- Spray bottle with 70% (vol/vol) ethanol
- Air-jacketed 37°C and 5% CO_2 incubator (VWR, cat. no. 10810–902)
- Biological safety cabinet (Labconco, cat no. 97000–862)
- Water bath
- Culture microscope (Olympus, cat. no. CKX41)
- EVOS FL Cell Imaging System (Life Technologies, mode AMF4300)
- Keyence fluorescence microscope, BZ-XY700 (Keyence, model BZ-X710)
- Confocal microscope (Leica, model TCS SP8)
- Stainless Steel Surgical Blade, Sterile, No 10 (Medicon, cat. no. 01.22.10)
- Sterile cell strainer (Corning, cat. no. 352340)
- Petri dishes (non-TC treated) 6 cm (Corning, product no. 430589)
- Hemocytometer
- Nylon 0.22μm nylon syringe filter (Celltreat, cat. no. 229775)
- 5702 series centrifuge (Eppendorf, cat. no. 022628102)
- 5% CO_2 tank with plastic hosing leading to culture hood. Setup can include multiple 3-way stops to split the hosing into multiple lines. To bubble CO_2 through solutions, attach a syringe filter to the end of the hosing and then attach the broken end of a 2 ml pipette and place tip into liquid. During dissociation, place a syringe filter on the end of the plastic tubing and set filter tip into small hole cut into the lid of the Petri dish (without contacting the solution) so that CO_2 can be blown over the digesting solution. If this setup is not available, solutions can also be equilibrated in the incubator.

For assembloid formation only:

- Sterile 1.5 ml microcentrifuge
- Scissors and/or razor blades

For cryosectioning and immunostaining only.

- Microslides superfrost plus (VWR, cat. no. 48311–703)
- Tissue Path Disposable Base Molds – 15 × 15 × 5 mm (Fisher Scientific, cat. no. 22–363-553)
- Humidified chamber (We use a home-made chamber made with a slide box and wet tissue paper)

- Elite PAP pen (Diagnostic BioSystems, cat. no. K039)
- Cover glasses – rectangles (Fisherbrand, cat. no. 22–266882)
- Cryostat (Leica, cat. no. CM1860)
- Sable brushes, #1, 1.5mm W x 9.5mm L (Ted Pella, cat. no. 11812)

Reagent Setup

Reconstitution and storage of growth factor and chemical stock solutions— Resuspend FGF-2, EGF, BDNF and NT3 growth factors in sterile 0.1% BSA, PBS to the desired stock concentration. Stock solution concentrations are as follows: FGF-2 stock solution, 20 μg/ml, and use at 10 ng/ml for hPSCs (1:2000) and 20 ng/ml for neural differentiation (1:1000); EGF, BDNF, NT3 stock solutions, 20 μg/ml, and use at 20 ng/ml (1:1000). Resuspend 25 mg dorsomorphin in 12.52 ml of sterile DMSO to obtain a 5 mM stock solution, and use at a final concentration of 5 μM (1:1000). Resuspend 10 mg SB-431542 in 2.60 ml 100% ethanol to obtain a 10 mM stock solution, and use at a final concentration of 10 μM (1:1000). Resuspend 10 mg IWP-2 in 4.29 ml of sterile DMSO to obtain a 5 mM stock solution; use at a final concentration of 5 μM (1:1000). Resuspend 5 mg SAG in 94.96 ml sterile DMSO to obtain a 100 μM stock solution; use at a final concentration of 100 nM (1:1000). Resuspend 5 mg Allo-P in 156.99 ml sterile DMSO to obtain a 100 μM stock solution and use at a final concentration of 100 nM (1:1000). Resuspend 50 mg RA in 166.42 ml sterile DMSO to obtain a 1 mM stock solution and further dilute stock solution to obtain 100 μM solution and use at a final concentration of 100 nM (1:1000). Aliquot stock solutions and store at –80°C. Once thawed, stocks can be kept at 4°C for up to two weeks.

General media guidance—Various media are required, compositions are as indicated below for each specific medium. When ready for use, aliquot required media into 50 ml sterile tubes and pre-warm in a water bath at 37°C for less than 20 minutes. Avoid cycles of refrigeration and warming. ! CRITICAL Add growth factors and small molecules immediately before use and only after pre-warming aliquoted media.

MEF media (for use in steps 1–2).

Prepare medium as detailed in the table below. This media can be stored for 1–2 weeks at 4°C.

Composition	Volume (500 ml)	Final concentration
DMEM (high glucose)	445 ml	
FBS	50 ml	10% (vol/vol)
GlutaMax	5 ml	1% (vol/vol)
NEAA	5 ml	1% (vol/vol)

hPSC media (for use in steps 5–16).

Prepare medium as detailed in the table below. This media can be stored for 1–2 weeks at 4°C in the absence of growth factors. Add growth factors (FGF2) just before use.

Composition	Volume (500 ml)	Final concentration	Comments
DMEM/F12	392.5 ml		
KSR	100 ml	20% (vol/vol)	
NEAA	5 ml	1% (vol/vol)	
GlutaMax	2.5 ml	0.5% (vol/vol)	
2-Mercaptoethanol	3.4 µl	0.1 mM	
FGF2 (20mg/ml stock)	250 µl	10 ng/ml	Add just before use.

Neural induction media (for use in steps 17–23, on days 0–5 of differentiation).

Prepare medium as detailed in the table below. This media can stored for 1 week at 4°C in the absence of growth factors. Add growth factors (Dorsomorphin, SB-431542 and IWP-2) just before use.

Composition	Volume (500 ml)	Final concentration	Comments
DMEM/F12	486.5 ml		
KSR	100 ml	20% (vol/vol)	
NEAA	5 ml	1% (vol/vol)	
GlutaMax	2.5 ml	0.5% (vol/vol)	
Penicillin/streptomycin	5 ml	1% (vol/vol)	
2-Mercaptoethanol	3.4 µl	0.1 mM	
Dorsomorphin	500 µl	5 µM	Add just before use.
SB-431542	500 µl	10 µM	Add just before use.
IWP-2	500 µl	5 µM	Only required for differentiation to hSS condition (Step 23, option B) from day 4 onward. Add just before use.

Neural differentiation media (for use in steps 22–23, on days 6 and onwards of differentiation).

Prepare medium as detailed in the table below. Store stock for up to 1 week at 4°C and add growth factors just before use if required for the culture.

Composition	Volume (~500 ml)	Final concentration	Comments
Neurobasal A	480 ml		
B27 supplement	10 ml	2% (vol/vol)	
GlutaMax	5 ml	1% (vol/vol)	

Composition	Volume (~500 ml)	Final concentration	Comments
Penicillin/streptomycin	5 ml	1% (vol/vol)	
EGF (20ug/mL stock)	500 µl	20 ng/ml	For hSS and hCS. Only from day 6–25. Add just before use.
FGF2 (20ug/mL stock)	500 µl	20 ng/ml	For hSS and hCS. Only from day 6–25. Add just before use.
BDNF (20ug/mL stock)	500 µl	20 ng/ml	For hSS and hCS. Only from day 25–43. Add just before use.
NT3 (20ug/mL stock)	500 µl	20 ng/ml	For hSS and hCS. Only from day 25–43. Add just before use.
IWP-2 (5mM stock)	500 µl	5 µM	Only for hSS conditions from day 6 to day 24. Add just before use.
SAG (100µM stock)	500 µl	100 nM	Only for hSS conditions from day 12 to day 24. Add just before use.
Allo-P (100µM stock)	500 µl	100 nM	Only for hSS conditions from day 15 to day 24. Add just before use.
RA (100µM stock)	500 µl	100 nM	Only for hSS conditions from day 12 to day 15. Add just before use.

Enzyme Stock Solution (required for dissociation of cells into a single cell suspension).

Prepare stock solution as detailed in the table below. Store stock for 2–3 months at 4°C.

Composition	Volume (200 ml)	Final concentration
ddH_20	170.4 ml	
10x EBSS	20 ml	1x
30% D(+)−Glucose	2.4 ml	0.46% (vol/vol)
1M $NaHCO_3$	5.2 ml	26 mM
50mM EDTA	2 mL	0.5 mM

Inhibitor Stock Solution (required for dissociation of cells into a single cell suspension).

Prepare stock solution as detailed in the table below. Store stock for 2–3 months at 4°C.

Composition	Volume (500 ml)	Final concentration
ddH_20	431 ml	
10x EBSS	50 ml	1x
30% D(+)−Glucose	6 ml	0.46% (vol/vol)
1M $NaHCO_3$	13 ml	25 mM

Dispase solution—Reconstitute dispase in hPSC media (without growth factors) to a stock concentration of 1.75 mg/ml. Aliquot stock solution and store at –20°C for up to one year. For hPSC passaging, thaw the dispase and dilute in hPSC media to a concentration of 0.875 mg/ml. For lifting hPSC colonies for sphere formation, dilute the stock solution to

0.35 mg/ml in hPSC media and warm in a water bath at 37°C for no more than 10 minutes. Working dispase solution can be kept at 4°C for up to 1 week.

Low Ovo (10×) (required for single cell dissociation only):

Add 3 grams of BSA to 150 ml DPBS and mix well. Add 3 grams of Trypsin inhibitor and mix to dissolve. Adjust pH to 7.4; this requires the addition of approximately 1ml of 1N NaOH. When completely dissolved bring to 200 ml with dPBS and filter through 0.22 μm filter. Make 1.0 ml aliquots and stored at –20˚C for up to 6 months.

High Ovo (10×) (required for single cell dissociation only):

Add 6 grams of BSA to 150 ml DPBS. Add 6 grams of Trypsin inhibitor and mix to dissolve. Adjust pH to 7.4; this requires the addition of at least 1.5 ml of 1N NaOH. If necessary, add NaOH until solution is no longer too acidic. Bring to 200 ml with DPBS. When completely dissolved, filter through 0.22 μm filter. Make 1.0 ml aliquots and stored at –20˚C for up to 6 months.

Paraformaldehyde—Prepare 4% (vol/vol) paraformaldehyde by mixing 10 ml 16% paraformaldehyde with 30 ml DI water, and store at 4°C for up to 1 week. ! CAUTION Paraformaldehyde must be handled in a safety cabinet and should be disposed of according to institutional guidelines.

Embedding solution

Prepare a 30%(wt/vol) sucrose solution by mixing 30 g of sucrose with PBS to a final volume of 100 ml. Store the 30% sucrose solution at 4°C for up to 2 weeks. Add 5 ml of OCT to 5 ml of 30% sucrose to make 1:1 embedding solution. Shake solution vigorously until OCT and sucrose have mixed together. Leave mixed solution overnight at 4°C to allow bubbles formed during the mixing process to disappear.

Blocking solution for immunostaining

Prepare the blocking solution for immunostaining by mixing 10% (vol/vol) normal donkey serum with 0.3% (vol/vol) Triton-X in PBS. Keep this solution at 4°C for up to 1 week.

PROCEDURE

MEF feeder seeding and initiation of hPSC cultures (TIMING: 2 hours)

1. Coat 10 cm cell culture dishes with 0.1% gelatin for at least 1 hour at RT (~24°C).
2. Thaw one vial of MEFs containing ~5 $\times 10^6$ cells by placing it in a water bath at 37°C until it is 80% thawed (and for no longer than 2 minutes). Briefly spin cells (at 200g for 4 minutes) and plate at a density of 1–2 $\times 10^6$ cells per 10 cm plate.
3. Next day, assess feeder quality before use; this will determine the quality of the hPSCs. Once MEFs are ready for plating hPSCs (after about 2 hours), wash the culture plates at least once with fresh PBS.

Passaging hPSCs on the MEF feeder layer (TIMING: 5 days)

4. Remove hPSC media from the fridge, aliquot the required amount (~12 ml per 10 cm plate), wrap the container's cap with parafilm to prevent contamination when putting it into the water bath, and warm in a water bath for up to 20 minutes.

5. Remove hPSC plate from incubator and look at it under a microscope. Whilst under the microscope mark the differentiated areas on the bottom of the plate using a marker. Differentiated colonies may appear to have a hole or pit in the center of the colony or a border of differentiated cells (Figure 3a).

6. After wiping with a 70% ethanol-sprayed paper towel, transfer the plates to the cell culture hood. Then use a pipet tip to carefully remove demarcated differentiated colonies from the plate.

7. Aspirate the media and add 5 ml of dispase (0.875 mg/ml in hPSC media) to each culture plate and place in cell culture incubator for 7 minutes or until hPSC colonies have lifted.

8. Transfer the suspended cells from a single plate into a 50 ml centrifuge tube. Use 5 ml of hPSC media with 10ng/ml FGF-2 and 10 μM Y-27632 to wash each 10 cm plate. Perform this step 3 times, adding the media to the appropriate 50 ml centrifuge tube following each wash.

9. Gently swirl the 50 ml centrifuge tubes to suspend harvested cells in 20 ml of hPSC media.

10. Transfer the hPSC media and suspended cells to a freshly prepared feeder plate (from step 3).

11. Gently swirl the plate and slide the plate in a back-and-forth motion on the shelf of the incubator to evenly disperse the cells.

12. Incubate cells in the cell culture incubator. Leave cells undisturbed for the first day after passaging, and replace media every day thereafter for 5–7 days. To replace media, warm up aliquoted fresh hPSC media in a water bath for up to 10 minutes at 37°C, aspirate old media off the plates and add 12 ml of fresh media per 10 cm culture plate.

    ! CRITICAL Replace hPSC culture media at approximately the same time each day.

13. Monitor hPSC colonies to determine when they are ready for spheroid formation. This will usually be around 6–7 days from the last passage (depending on the hPSC line), and colonies should measure around 1.5 mm in diameter. See Figure 3a for examples of hPSC colonies of appropriate, and inappropriate, morphology. When cultures are ready, proceed to the next step. Alternatively, continue to passage hPSCs every 5–7 days (depending on the growth rate of each line) by repeating steps 1–12, however bear in mind that ideally, hPSCs should not be kept beyond passage 40.

! CRITICAL It is essential for the colonies to be large to generate spheroids (i.e., just prior to the time at which colonies would be passaged, or 1–2 days later). If colonies are too small, allow more time for growth prior to subsequent steps. Avoid using hPSC colonies that touch each other because they will detach as large clusters together with the MEF layer. Avoid hPSC colonies growing too big and differentiating in the center.

Suspending hPSC colonies to form spheroids (TIMING: 2–3 hours)

14. Remove the hPSC media from the culture plates. Detach hPSC colonies by incubating them with 0.35 mg/ml dispase (dissolved in hPSC media; 5 ml per plate) for 30–45 minutes at 37°C, 5% CO_2. Check the status of the detachment after 20 minutes and then every 5 minutes thereafter. Different lines will require different durations of dispase treatment. Plates can be gently moved (back and forth) at the end of the incubation period to facilitate the detachment of curled hPSC colonies from the feeders. Almost all feeders should stay attached to the 10 cm plate, while the hPSC colonies should come off. Intact colonies should curl up and lift off the plate (Figure 3b).

15. Gently transfer the floating colonies with a pre-wet 25 ml pipette to a 50 ml Falcon tube. Once the colonies settle at the bottom of the tube, aspirate the media and gently wash the colonies two times with 20 ml of warm hPSC media (without growth factors). ! CRITICAL Do not centrifuge. Let the colonies settle at the bottom of the Falcon tube. TROUBLESHOOTING

16. Gently resuspend 30–50 suspended colonies in 12 ml neural induction media (the media should also contain 5 μM dorsomorphin (DM), 10 μM SB-431542 (SB) and 10 μM Y-27632) and transfer to a 10 cm ultra-low-attachment plate. Denote this day as day 0. Place in incubator at 37°C, 5% CO_2 for 48 hours. Keep the spheroids in the incubator on day 1 without any media change to allow the spheroids a day to recover. ! CAUTION It is important to distribute the spheroids evenly once the plate is placed in the incubator to avoid clustering and fusion of colonies at the center of the plate.

Neural induction (TIMING: 6 days)

! CRITICAL Minimize the overall time that the spheroids are kept outside of the incubator at all stages of differentiation.

17. Day 2: Following the ~48 hours of incubation, i.e. on day 2, replace media with 12 ml neural induction media (with DM and SB but without Y-27632). To change media, gently transfer the spheroids to a 50 ml Falcon tube and carefully aspirate the media once they settle Incubate cells for a further 24 h.

18. Day 3: Replace media as described in step 17 and incubate cells a further 24h.

TROUBLESHOOTING
See Table 2 for troubleshooting guidance.

19. Day 4: If you plan to differentiate hSS (when you reach Step 22, Options B, C), replace media as described in step 17, but include 5 μm IWP-2 in the media in addition. If you plan to generate hCS, replace media as before. Incubate cells a further 24h.

    ! CRITICAL STEP IWP-2 should not be thawed at room temperature as it will precipitate. Frozen aliquots should be placed directly in the water bath before use.

20. Day 5: Replace media as described in step 19. Incubate cells a further 24h.

Patterning and differentiation (TIMING: up to 37 days)

21. From day 6 onwards, replace media daily with neural differentiation media (NM) in place of the spheroid media. Use NM supplemented with 20 ng/ml FGF2 and 20 ng/ml EGF. Also include 5 μm IWP-2 in the media if you are generating hCS. Change media daily on days 6–11. At day 6 neural spheroids should be about 0.3–0.5 mm in diameter Figure 3c). Media can be changed as described in step 17 or by gently tilting the plate (allowing the spheroids to settle) and removing the remaining media.

22. From day 12 onward the media requirements for generating hCS or hSS deviate further. To generate hCS follow option A (**see** Figure 2c **for detailed protocol timeline**). To generate hSS follow option B (**see** Figure 2c **for detailed protocol timeline**). To generate hSS with more activity visible by calcium imaging[19] (hSS-ISA), follow option C. After neural spheroids grow larger (more than 2–3 mm in diameter), no more than 30 spheroids should be maintained per 10 cm plate.

    ! CRITICAL Neural spheroids tend to fuse to each other as they get larger (Figure 3d). When changing media, be sure to separate them by either pipetting up and down gently with a 10 ml pipette or by using a P10 pipette tip to separate them when they are bigger. If spheroids fuse during later timepoints (after ~day 50), it may greatly compromise the outcome. TROUBLESHOOTING

Option A: Generation of hCS

i. From days 12–15, continue to replace media daily with neural differentiation media (NM) supplemented with 20 ng/ml FGF2 and 20 ng/ml EGF.

ii. From day 16 onward, change media every other day until day 25. Continue to use neural differentiation media (NM) supplemented with 20 ng/ml FGF2 and 20 ng/ml EGF.

iii. From day 25 to day 43, replace FGF-2 and EGF with 20 ng/ml BDNF and 20 ng/ml NT3. Perform media changes with 14–15 mL supplemented NM per 10 cm plate every 2–3 days.

Option B: Generation of hSS

i. From day 12 to day 23, supplement neural differentiation media (already containing 20 ng/ml FGF2, 20 ng/ml EGF and 5 μm IWP-2) with 100 nM SAG. Perform media changes with 14–15 mL supplemented NM per 10 cm plate every day.

! CRITICAL Note, early hSS can be smaller in diameter in comparison to early hCS in the 12–20 days of differentiation.

ii. From day 25 to day 43, replace FGF-2 and EGF with 20 ng/ml BDNF and 20 ng/ml NT3. Perform media changes with 14–15 mL supplemented NM per 10 cm plate every 2–3 days.

Option C: Generation of hSS-ISRA.

i. On days 12, 13 and 14, supplement neural differentiation media (with IWP-2) with 100 nM SAG. Perform media changes with 14–15 mL supplemented NM per 10 cm plate every day.

ii. From days 15–23 add 100 nM Allopregnanolone (AlloP) and from day 12–15 add 100 nM retinoic acid (RA) in addition to the IWP-2 to the neural induction media (Fig. 2c). CAUTION Retinoic acid is air and light sensitive. Minimize light exposure.

iii. From day 25 to day 43, replace FGF-2 and EGF with 20 ng/ml BDNF and 20 ng/ml NT3. Perform media changes with 14–15 mL supplemented NM per 10 cm plate every 2–3 days.

Maintenance and long-term culture (TIMING indefinite)

23. From day 43 onwards, perform media changes with 17–18 ml NM without growth factors every four days. With careful sterile technique, spheroids can be maintained for many months in culture, if desired (Figure 3d). Carry out any additional procedures at time points determined by your experiment. Examples of additional procedures that can be carried out on the spheroids include the generation of forebrain assembloids (option A), dissociation into single cell suspension in preparation for further assays (option B), cryosectioning and immunolabelling (option C), calcium imaging (option D) and optical clearing by iDISCO (option E). Bear in mind that if you wish to assemble forebrain spheroids (option A) that this is most successful if the combined size of the spheroid is less than 6 mm (between days 60–90 of culture). TROUBLESHOOTING

Option A: Generating Forebrain Assembloids using hCS and hSS (TIMING 3–7 days)

i. For assembly of forebrain spheroids, transfer one hCS and one hSS into a 1.5 ml microcentrifuge tube that is resting in a standard microtube rack (Figure 4f). Denote the day of assembly as day 0. The size of the spheroids to be assembled should not exceed 3 mm, as they are challenging to fuse once they are too large. ! CRITICAL Once in the tube, make sure that there are no bubbles underneath the

spheroids and that they rest next to each other in contact at the bottom of the tube and not stacked up vertically

ii. Incubate the spheroids for at three days in 1ml neural media, completely replacing the media on day 2. ! CAUTION As the assembly interface is fragile at this stage, perform the media change in the tube very carefully. TROUBLESHOOTING

iii. Check that fusion is complete. Fusion is considered complete when the two spheroids are inseparable with gentle shaking of the Eppendorf tube (Figure 4g). After fusion, use a P1000 pipette with the tip cut for a larger bore opening to transfer the assembled hSS–hCS back into ultra low-attachment 10 cm plates. If assembly is carried out between days 60 and 90, when assembly of hSSs and hCSs is most efficient, assembly is usually complete after 3–4 d. Earlier time points are also permissive. Assembly of hSS-hCS at later stages of differentiation (after day 90) may take longer to assemble (up to 7 days). TROUBLESHOOTING

Option B: Dissociating into single cell suspension (TIMING: 2–4 hours)

i. Aliquot enzyme stock (use 10 ml per dissociation dish, which should correspond to about 15 early stage spheroids or 7 late stage spheroids, plus an extra 2ml) into a 50 ml Falcon tube, and bubble 5% CO_2 through solution at RT until the solution turns from red to orange.

ii. Once warmed and orange in color, add papain to the enzyme stock (for neural spheroids up to day 200, use 30 units/ml, and after that differentiation stage use 50 units/ml).

iii. Add 0.0036–0.0042 grams of L-cysteine per 20 ml of enzyme stock and put solution into 34–37°C water bath. Warm up solution mixture for at least 15 minutes before proceeding to the digestion step (vi). In the meantime, proceed to the next step.

iv. Transfer spheroids to a 6 cm Petri dish, remove any residual media and use a no. 10 scalpel blade to chop into < 1 mm^3 pieces. Finer pieces are preferable (this will require at least 30 seconds of chopping).

v. Use 2 ml of enzyme stock to wet a 0.22 μm filter, then filter 10 ml enzyme stock into each Petri plate containing the finely chopped spheroids. Add 200 μl DNase to each Petri plate and swirl dish to mix.

vi. For the papain digestion, put the Petri dishes on a 34°C heat block on top of a wet paper towel. Attach a hose from a CO_2 tank to a 0.22 μm syringe filter and place the filter tip into a small hole cut into the lid of the Petri dish to flow 5% CO_2 over the enzyme stock solution with tissue pieces. ! CRITICAL The filter tip should not touch the solution to ensure that gas is not bubbling into the liquid but rather blowing over the top.

vii. Gently Shake the Petri plate every 15 minutes. Digest for 70–100 minutes. Digestion time should be increased with the age of spheroids to a maximum of 100 minutes for spheroids older than 250 days. During the digestion proceed with the next step.

viii. During digestion, prepare Low Ovo and High Ovo solutions by diluting 10× low and high Ovo in inhibitory stock solution to make 1x final concentrations of each. Trituration will require ~10 ml low ovo solution and 1 ml high Ovo solution per dissociation dish.

ix. After digestion, transfer the enzyme solution with tissue pieces into a 15 ml Falcon tube. Add 4–5 ml low Ovo solution. Spin at 100 xg for 2 minutes until tissue chunks settle at bottom. Aspirate and discard the supernatant.

x. Add 1ml of low Ovo into the tube, and use a P1000 pipette to triturate 10–20 times. The solution should become cloudy. Be careful to avoid introducing bubbles by not lifting the pipette out of the solution during trituration. After the first round of trituration, allow chunks to settle to the bottom, and then transfer single cells (supernatant) to a new tube.

xi. Repeat trituration process with additional aliquots of 1ml of low Ovo solution and 10–20 triturations each time. It may take 2–3 rounds until tissue chunks are dissolved. With each successive round, be more aggressive with trituration.

xii. Once all tissue chunks have dissolved, use a P1000 pipette to carefully layer 1 ml of high Ovo solution under the single cell suspension. This should lead to a clear layer of liquid beneath a cloudy cell suspension.

xiii. Spin cells down through the high Ovo at 100 g for 5 minutes at RT. Aspirate and discard the supernatant. A cell pellet should be visible at the bottom of the Falcon tube.

xiv. Resuspend cell pellet in media of choice. Single cell suspensions can be plated down for cell culture, time-lapse imaging, calcium imaging, immunohistochemistry, or can then be immunopanned or FACS separated.

Option C: Cryosectioning and immunostaining (TIMING: 3–5 days)

i. At time point of choice, transfer spheroids to a 1.5 ml microcentrifuge tube using a cut P1000 tip. Gently remove media and briefly wash with 1ml of PBS. Remove PBS and add 500μl of cold 4% PFA. Leave spheres to fix for at least 30 minutes at 4°C. The optimal fixation time varies depending on the antibody being used and the age and size of the spheres. We thus recommend fixation trials be undertaken to optimize the optimal fixation required for specific antibody stainings; e.g. for membrane antigens we have found fixation for shorter periods of time (~2 hours) is optimal whereas for nuclear antigens, optimal results are seen following a longer fixation (up to overnight). CRITICAL Use the same fixation and embedding protocol when quantifying across hCS or hSS derived from multiple hPSC lines and differentiations. Variation in fixation and sample processing protocol can lead to variability in antibody staining.

ii. After fixation, gently remove PFA and perform three 15 minute washes with PBS at RT.

iii. Add 1 ml of 30% sucrose to facilitate cryo-protection. Leave spheres in this solution at 4°C until they sink (~24–48 hours). During this time, prepare the embedding solution (1:1, OCT: 30% sucrose, see reagent setup section).

iv. To embed spheres, use square disposable molds that have been filled with embedding solution. ! CRITICAL To avoid creating bubbles while filling the mold, use a P1000 pipette where the tip has been slightly cut to widen the opening.

v. Place spheres in mold using a P1000 cut pipette tip. Place 4–10 spheroids in the mold, always making sure to mark their locations. If spheres move while embedding, use a P20 tip to gently move them around the embedding solution. ! CRITICAL Avoid carrying excess 30% sucrose when transferring spheres

vi. Place the mold on ice for 20 min and allow spheres to sink to the bottom.

vii. Snap freeze spheres by placing the mold directly on dry ice. Once completely frozen, store molds at −80°C. PAUSE POINT Frozen molds can be stored indefinitely at –80°C until ready to cryo-section.

viii. When ready for sectioning, remove mold from –80°C and allow it to increase in temperature to –20°C by placing it in the cryostat chamber for ~30 minutes before sectioning.

ix. Remove block from mold, paste on specimen stage using OCT and section using standard techniques. Use a brush to prevent crumbling of the sections. We recommend that hCS or hSS be cryosectioned at 10–15 μm thickness. Thicker sections are difficult to quantify because of the overlapping nuclei and high density of cells, especially in proliferative areas. However, if using a confocal microscope, thicker sections may be adequate.

x. Collect sections on superfrost plus slides. PAUSE POINT Slides can be stored at –80°C until ready to use.

xi. For immunostaining, remove slide from –80°C and leave at RT to thaw for 5–10 minutes.

xii. Wash slide once with PBS to remove OCT:sucrose.

xiii. Remove PBS and use a hydrophobic PAP pen to draw a circle around sections. Let pen dry and wash with PBS again to remove excess. From this step onwards, add all solutions within the circle.

xiv. Block for 1 hour at RT with 10% NDS, 0.3% Triton-X, PBS. Note that the optimal concentration of NDS and Triton-X to use may vary according to antigen of interest. ! CRITICAL Perform immunostaining steps in a humidified chamber to avoid sections drying out.

xv. Add primary antibodies diluted in block solution at 4°C overnight. Some examples of antibodies and concentrations that we have used successfully are given in Table 1. ! CRITICAL Some antibodies might need longer incubation periods and so further optimization may be required.

xvi. Perform three 15–minute washes at RT with PBS.

xvii. Add appropriate secondary antibodies diluted in block solution for 1 hour at RT. ! CRITICAL Protect sections from light during this and following steps.

xviii. Perform three 15–minute washes at RT with PBS.

xix. Add Hoechst solution (1/10.000 in PBS) for 5–10 minutes at RT.

xx. Remove excess Hoechst by washing with PBS.

xxi. Remove PBS, let sections air dry for 5–10 min, and mount by adding one drop of Aquamount solution on top of sections and placing a coverglass on top.

xxii. Use a fluorescent microscope to visualize immunostained sections. See Figure 4 for representative immunostainings of hCS and hSS with dorsal and ventral-specific domain markers.

! CRITICAL Ideally, cryosections for quantification should be sampled throughout the thickness of the spheroid. Avoid quantification of cryo-sections that are not at least 50 μm apart (5 cryo-sections apart if cutting at 10 μm) to prevent sampling the same cells. Avoid extreme edges of the hCS or hSS since they are not likely to be representative and important cell types might be missed that are usually present only in the deeper parts of the spheroids.

Option D: Calcium imaging (TIMING: 2–6 hours)

i. Incubate intact spheroids with 10 μM Fluo-4 acetoxymethyl ester (Fluo-4AM; Invitrogen) diluted in neural media for 30 minutes at 37°C, 5% CO_2 followed by a 15–minute wash.

ii. Using an appropriate imaging system, record spontaneous calcium activity for several minutes (one frame every 8–10 seconds) in one 10 μm z-stack plane. Maintain spheroids in neural media during recordings. We do not perfuse during calcium imaging sessions to minimize drift.

iii. Export fluorescence intensity (F) as mean gray values in the ImageJ software (https://imagej.nih.gov/ij/). Signal decay should be controlled by subtracting the mean fluorescence of the background (F_b). To estimate changes in intracellular calcium, ΔF is computed as $(F_{cell} - F_b)/F_0$, where F_0 represents the minimum F value per cell across the entire duration of recording from which F_b was subtracted.

Option E: Optical clearing of intact spheroids by iDISCO (TIMING: 12 days)

i. Fix spheroids with 4% PFA for 3 hours at 4°C. Note that this incubation time was optimized for 50–150 day old hCS, hSS or forebrain assembloids. However,

given that the size of spheroids after this age does not significantly change, this fixation time is likely to be suitable for older spheroids as well. If using younger spheroids (up to 25 days old), incubation times may be shortened. Keep in mind, however, that iDISCO clearing tends to result in a shrinking of tissue, so using early spheroids may make handling challenging.

ii. Perform three 15 minute-long washes at RT with PBS.

iii. Transfer the spheroids to 4 ml glass vials.

iv. Dehydrate spheroids with a day-long methanol (MetOH) dilution series at RT (20%, 40%, 60%, 80% and 100% MetOH/ PBS – 30 minutes per step)

v. Incubate spheroids in 5% H_2O_2/100% MetOH overnight at 4°C.

vi. The following day, rehydrate with a reverse MetOH dilution series (80% MeOH/ PBS, 80% 60%, 40%, 20%, 0% and 0% MeOH/0.2% TX/PBS – 30 minutes per step).

vii. Incubate spheroids overnight in 0.2% Triton-X, 20% DMSO, 0.3 M Glycine/PBS at 37°C.

viii. Block spheroids with 0.2% Triton-X, 10% DMSO, 6% goat serum/PBS at 37°C for 2 days. ! CRITICAL Perform this and all following steps in closed tubes filled to the top to prevent oxidation of tissue.

ix. Incubate spheroids with 0.2% Tween–20 for one hour.

x. Incubate spheroids in 10 μg/ml Heparin/PBS (PTwH) for 1 hour.

xi. Incubate spheroids with desired primary antibodies (e.g., anti-GFAP, DAKO z0334, 1:1000 dilution; anti-GFP, GeneText, 1:1000; anti-SOX2, Cell Signaling, 1:500) for 2 days in PTwH with 5% DMSO and 3% goat serum at 37°C.

xii. Undertake a day-long wash series with PTwH (30 minute to 1 hour steps for at least 5 hours: 10 min: PTwH, 15 min: PTwH, 30 min: PTwH, 1 hour: PTwH, 2 hours: PTwH).

xiii. Dilute secondary antibodies in PTwH and 3% goat serum and incubate for an additional two days at 37°C.

xiv. Wash off excess secondary antibody by washing for 2 hours with PTwH. Repeat three times so spheroids have been washed for at least 8 hours.

xv. Incubate spheroids in 50% tetrahydrofuran (THF)/H_2O overnight at RT.

xvi. Clear the spheroids with a three-step tetrahydrofuran (THF) series (1 hour 80%/ H_2O, 1 hour 100% THF, 1 hour 100% THF) at RT. Neural spheroids should be cleared within only a few minutes of benzyl ether incubation.

xvii. Image cleared tissue. When imaging cleared tissue, it is critical to use a chamber that is resistant to the organic solvents used in steps 15 and 16. For this purpose, we 3D printed a chamber using semi-clear acrylic type plastic from Projet at the Stanford 3D Printing Core and followed instructions that can be found at https://

idisco.info/idisco-protocol/. If using this chamber, use silicone sealant to fix a rectangular cover glass on the bottom of the 3D-printed chamber. When spheres are cleared, add them to the chamber with DBE. ! CRITICAL Make sure the chamber is completely sealed on to the cover glass to avoid any DBE leaking on to the microscope. We recommend visiting https://idisco.info/ for any additional troubleshooting tips.

Option F Live imaging of cell migration and calcium signaling (TIMING: 2–24 hours)

i. Transfer fused hCS-hSS to a well of a 96–well plate (glass-bottom plates, Corning) in 200 μl of neural media. Due to the limited amount of media that can be included in a 96 well plate, avoid putting more than one brain assembloid per well.

ii. Place 96-well plate into inverted microscope with a motorized stage under environmentally controlled conditions (37°C, 5% CO_2).

iii. Maintain assembled spheroids in an environmentally controlled chamber for 30–60 minutes prior to imaging to let them acclimatize and settle. Once settled, forebrain assembloids will not move during the imaging session providing any movement in the room is kept to a minimum.

iv. Set up imaging positions so that up to ~8 fields with z-stacks (corresponding to 8 assembled hSS-hCS) can be imaged in a sequential manner, with each position or frame being imaged at a rate of 15–20 minutes/frame. Our Z-stacks normally span a depth of 50–150 μm and have a step size of 1.5–2.5 μm. We have found that hss-hCS can be imaged for up to ~18 hours after which time imaging needs to be paused so media should be carefully changed before continuing imaging. On our live imaging system (Leica SP8 confocal microscope), we use less than 10% laser intensity per laser line with the 10x dry objective to avoid photo-bleaching and photo-toxicity when imaging 4–8 fields at a time. The laser power density should be empirically determined in other imaging systems.

v. If spheroids drift and this impacts image analysis, correct image timeseries for drift computationally. We have found that the image registration plugins from ImageJ (e.g. Linear stack alignment with SIFT) are generally sufficient to correct for minor to moderate drift. However we discard sessions with major drift due to handling. Occasional drifting of spheroids may occur as they are not embedded during imaging sessions.

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BOX 1. Viral Labelling of hCS and hSS (TIMING: 6–10 days)

Additional reagents and materials

- HEK 29T cells (ATCC cat. no. CRL-3216)
- Lenti-X concentrator (Clontech, cat. no. 631232)
- Packaging plasmids (Takara cat. no. 631275)
- Target plasmid
- Opti-MEM reduced serum media (Life Technologies, cat. no. 31985–062)
- Lipofectamine 2000 transfection reagent (Life Technologies, cat. no. 11668019)
- AAV-DJ-hSyn::YFP or AAV-DJ-hSyn::mCherry (for labeling neurons in hCS)
- Lenti-Dlxi½::eGFP (for labeling cortical GABAergic interneurons)
- Lenti-GFAP::eGFP (for labeling astrocytes in hCS and hSS)

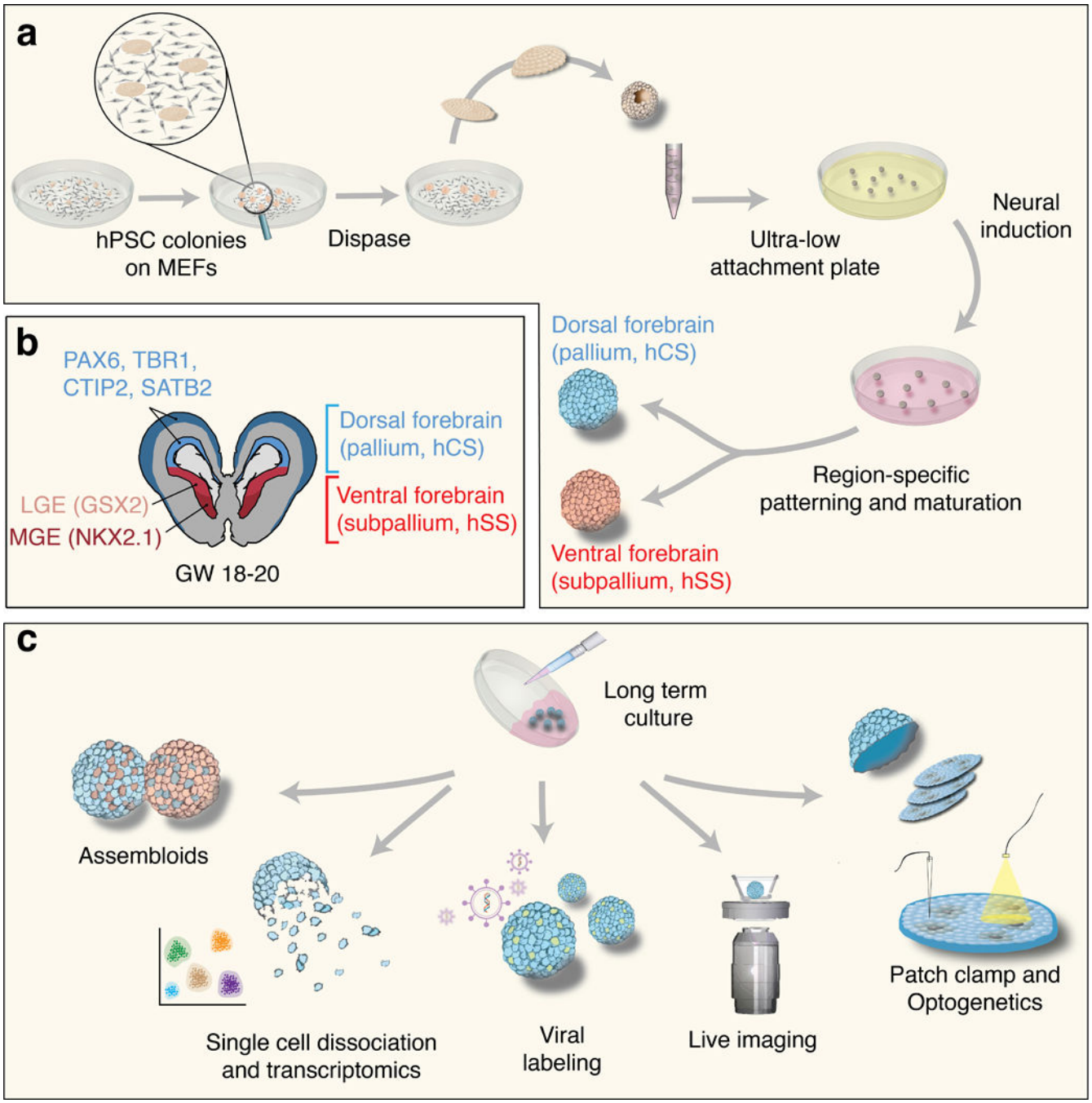
Additional equipment

- Parafilm (VWR, cat. no. PM-999)

Procedure

1. hCS and/or hSS can be fluorescently labeled using viruses prior to assembly. We obtain AAVs from the Stanford Gene and Vector Core. AAV-DJ is generally our serotype of choice. However, we have successfully used other AAV serotypes[21]. We make lentiviruses in house using standard protocols[47]. Briefly, transfect target plasmid and third-fourth generation packaging plasmids in HEK 293T cells using Lipofectamine 2000 (Life Technologies, cat. no. 11668019). After collecting the supernatant, concentrate the lentivirus by centrifugation with Lenti-X concentrator (Clontech, cat. no. 631232).
2. For labeling spheroids, add the virus to a 1.5 ml microcentrifuge tube containing 1–2 spheroids in 250 µl of NM. Agitate the tube gently and incubate overnight in an incubator at 37°C, 5% CO_2 ! CRITICAL STEP The optimal titer for each virus needs to be empirically determined.
3. On the next day, add an extra 800 µl NM media to the mix and incubate for 24 hours. On day 2, transfer the spheroids back to 10 cm plates. Expression is usually seen 6–10 days post-exposure. TROUBLESHOOTING

Timing
• Steps 1–3, MEF feeder seeding and initiation of hPSC: 2 hours.
• Steps 4–13, Culture of hPSCs on the MEF feeder layer: 4–7 days (1 hour per day).
• Steps 14–16, Suspending hPSC colonies to form spheroids: 2–3 hours, Day 0.
• Steps 17—20, Neural induction: 6 days, Days 1–6. (1 hour per day)
• Steps 21–22, Patterning and differentiation: Days 7–43. (30 minutes per media change)
• Step 22, Option A: Generation of pallial forebrain-specific spheroids (hCS), Days 25–43. (30 minutes per media change)
• Step 22, Option B: Generation of subpallial forebrain-specific spheroids (hSS), Days 4–43. (30 minutes per media change)



• Step 22, Option C: Generation of hSS-ISRA, Days Option C: Generation of hSS-ISRA. Days 4–43. (30 minutes per media change)
• Step 23, Maintenance and long-term culture: Days 44–850+. (30 minutes per media change, every 4 days)
• Step 23, Option A: Generating forebrain assembloids using hCS and hSS: 3–7 days.
• Step 23, Option B: Dissociating into single cell suspension: 2–4 hours.
• Step 23, Option C: Cryosectioning and immunostaining: 3–5 days.
• Step 23, Option D: Calcium imaging: 2–6 hours.
• Step 23, Option E: Optical clearing of intact hCS or hSS by iDISCO: 12 days.
• Step 23, Option F: live imaging of cell migration and calcium imaging: 2–24 hours.
• Box 1, Procedure for viral labelling of hCS and hSS: 6–10 days.

Figure 1. General schematic for generating human forebrain spheroids from hPSC.

(**a**) Scheme illustrating the main stages of the method for generating dorsal (hCS) and ventral (hSS) forebrain spheroids from hPSCs.

(**b**) Coronal section from GW18–20 human brain outlining dorsal (pallial) and ventral (subpallial) markers and their domains.

(**c**) Examples of applications and functional assays that can be carried out using region-specific and assembled neural spheroids.

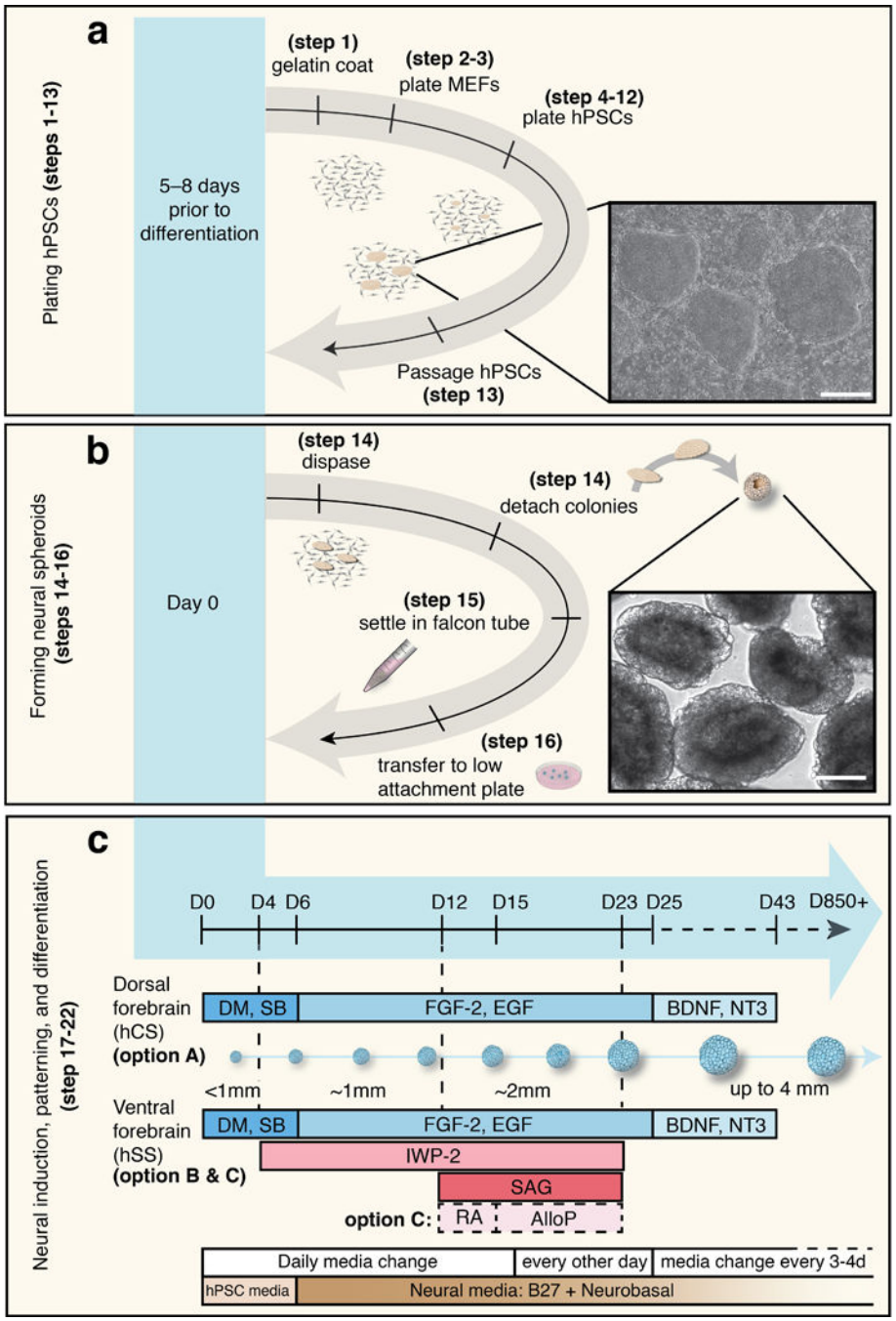


Figure 2. Outline of the human forebrain spheroid protocol.

(**a**) Recommendations for growing hPSC colonies prior to aggregation and spheroid formation. Representative image of ideal hPSC colonies prior to passaging or enzymatic lifting to form spheroids. Scale bar, 600 μm.

(**b**) Details of how to lift hPSC colonies to form spheroids using the enzyme dispase. Representative image of colonies immediately following dispase treatment or after transfer to ultra-low attachment plates. Scale bar, 150 μm.

(**c**) Neural induction and patterning protocols and timeline for generating dorsal and ventral forebrain-specific 3D cultures, including hSS-ISRA (option C that includes exposure to RA and AlloP).

The results shown here are from experiments using hPSC that conformed to institutional and federal regulations. IRB panel approval and appropriate informed consent were obtained for these studies.

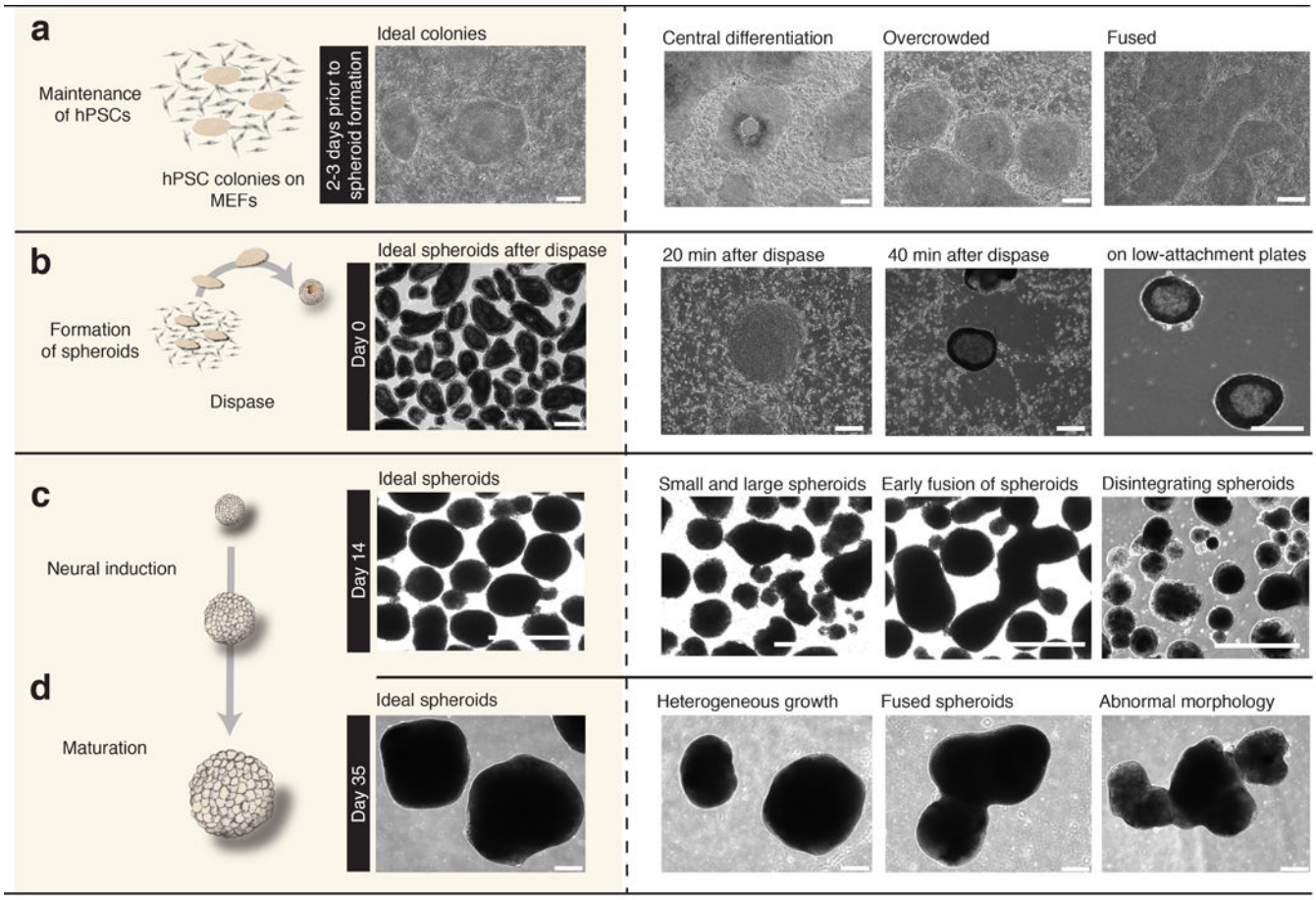


Figure 3. Images of forebrain spheroid formation.

Images of spheroids growing optimally (left hand images) and suboptimally (right hand images) are shown.(**a**) Representative images of hiPSC colonies grown on MEFs. Note that ideal colonies are large and sparsely distributed without evidence of central differentiation. Scale bar, 500 μm.

(**b**) Representative images of spheroid formation during and immediately following dispase treatment. Note that intact colonies are separated without dislodging the layer of MEFs. Scale bar, 500 μm.

(**c**) Representative images of ideal and abnormal neural spheroids during the neural induction period (days 3–14). Scale bar, 500 μm.

(**d**) Representative images of ideal and abnormal neural spheroids during the maturation phase (after day 25). Note the accelerated growth in size of neural spheroids at this stage. Scale bar, 500 μm.

The results shown here are from experiments using hPSC that conformed to institutional and federal regulations. IRB panel approval and appropriate informed consent were obtained for these studies.

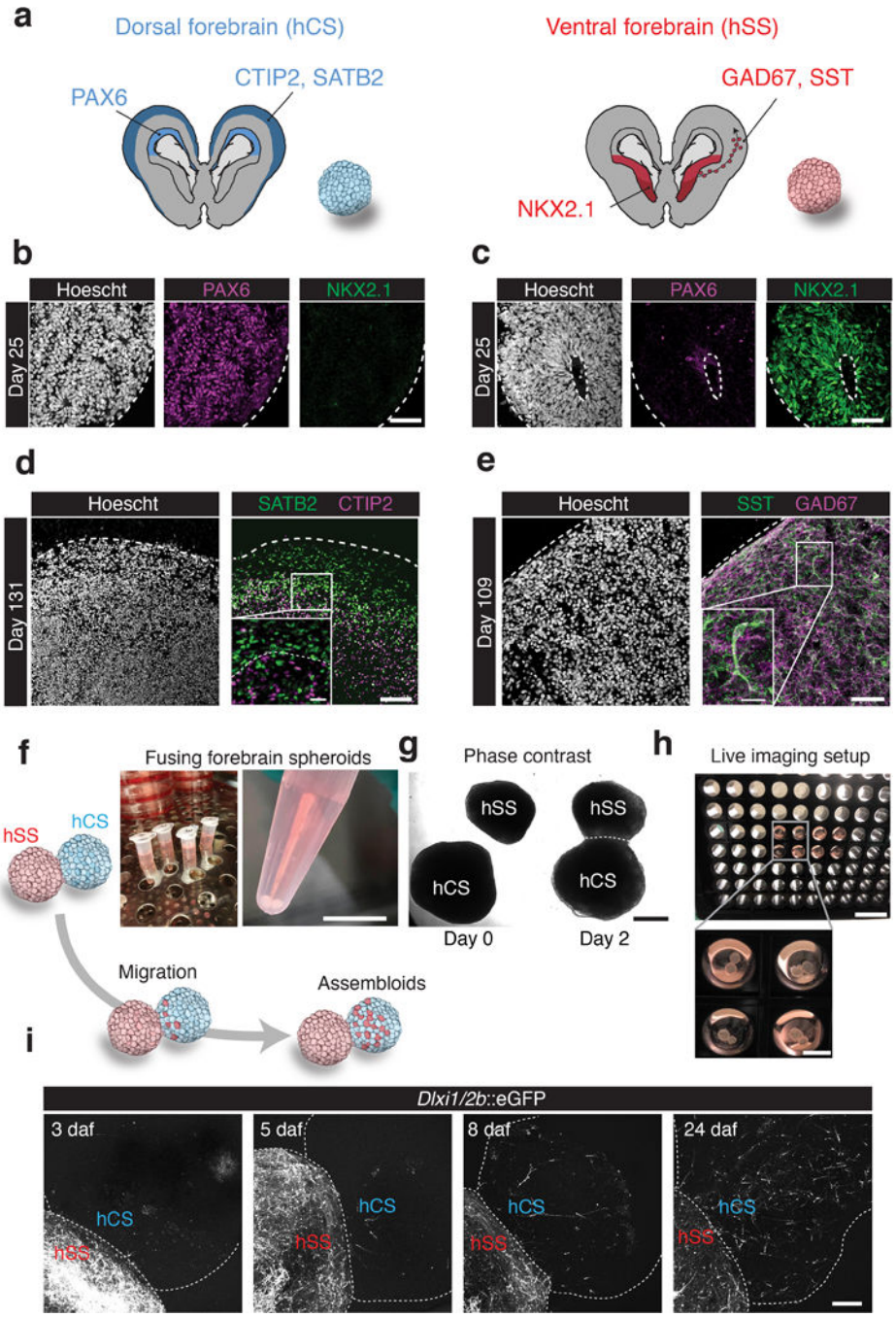


Figure 4. Expression of region-specific markers and fusion of dorsal and ventral neural spheroids into assembloids.

(**a**) Scheme illustrating expression patterns of dorsal and ventral forebrain markers in a coronal slice of GW18–20 human fetal brain.

(**b**) Dorsal forebrain marker expression (PAX6) and ventral forebrain marker expression (NKX2.1) in pallial (hCS) and subpallial (**c**) spheroids (hSS) at day 25 of differentiation. Scale bar, 50 μm.

(**d**) Pattern of expression of cortical markers in hCS at day 131 of differentiation *in vitro*. Scale bar, 100 μm. Inset shows expression pattern of CTIP2 (also known as BLC11B) and SATB2. Scale bar, 50 μm.
(**e**) Pattern of expression of ventral forebrain markers in hSS at day 109 of differentiation. Scale bar, 50 μm. Inset shows details of GABA-ergic neuron. Inset scale bar, 12 μm.
(**f**) Scheme and photos showing how spheroids are fused in the bottom of a 1.5 ml Eppendorf on day 0 of forebrain assembloid formation. Eppendorf tubes are held upright in a standard microtube rack. Inset scale bar, 1 cm.
(**g**) Phase contrast image of forebrain assembloids at day 0 and at day 2. Scale bar, 500 μm.
(**h**) Images following transfer of forebrain assembloids to a 96-well plate for live imaging. Scale bar, 4 cm. Inset scale bar, 1 cm.
(**i**) Use of forebrain assembloids to monitor migration of virally labeled (lentivirus, Dlxi½b::eGFP) GABA-ergic neurons from hSS into hCS throughout first 24 days after fusion (daf). Scale bar, 200 μm.
The results shown here are from experiments involving the use of hPSCs that conformed to institutional and federal regulations. IRB panel approval and appropriate informed consent were obtained for these studies.

TABLE 1.

Markers that can be used to characterize hPSC-derived 3D spheroids.

Marker	Temporal expression	Spatial expression and specificity	Antibody (optimal Dilution)
SOX2	Throughout differentiation	Present in pluripotent stem cells, neural progenitors and astrocytes in both hCS and hSS	Cell Signaling Technologies, SOX2 (D6D9), cat. no. 3579S (1: 500)
PAX6	From ~day 20	Early neuroectoderm progenitors and dorsal pallium progenitors	DSHB, cat. no. PAX6 (1:250)
TBR2	From ~day 50	Intermediate progenitors	Abcam, cat. no. ab757520 (1/300)
HOPX	From ~ day 50	Outer radial glia	Santa Cruz, cat. no. sc-398703 (1:500)
TBR1	From ~ day 50	Deep layer neurons in hCS	Abcam, cat. no. ab31940 (1: 500)
CTIP2	From ~ day 50	Deep layer neurons in hCS, but also expressed in hSS neural cells	[25B6] Abcam, cat. no. ab18465 (1: 300)
SATB2	From ~ day 100	Superficial layer neurons in hCS	[SATBA4B10] Abcam, cat. no. ab51502 (1: 400)
NKX2.1	From ~ day 25	Ventral forebrain progenitors (hSS)	Santa Cruz, cat. no. sc-13040 (1: 200)
GAD67	From ~ day 50	GABAergic neurons in hSS; sporadic expression in hCS	Millipore, cat. no. MAB5406 (1: 1000)
GABA	From ~ day 50	GABAergic neurons in hSS; some hCS cells can also express it transiently	Sigma-Aldrich, cat. no. A2052 (1: 1000)
SST	From ~ day 50	Interneuron subtype in hSS	Millipore, cat. no. MAB354 (1: 200)
PV	From ~ day 200	Interneuron subtype in hSS	Swant, cat. no. PV27 (1: 6000), Millipore, cat. no. MAB1572 (1: 1000)
CR	From ~ day 50	Interneuron subtype in hSS; sporadic expression in hCS	Swant, cat. no. CR7697 (1: 1000)
CB	From ~ day 50	Interneuron subtype in hSS; sporadic expression in hCS	Swant, cat. no. CB38 (1: 1000)
NEUN	From ~ day 75	Neurons in both hCS and hSS	Millipore, cat. no MAB377 (1: 500)
MAP2	From ~ day 30	Neurons in both hCS and hSS	Synaptic Systems, cat. no. 188004 (1:10.000)
GFAP	From ~ day 50	Astrocytes in both hCS and hSS, also marks radial glia	DAKO, cat. no. Z0334 (1: 1000)

TABLE 2.

Troubleshooting Table.

Step	Problem	Possible reason (s)	Solution
15	Spheroids do not form	– Differentiated hPSC colonies – MEF quality inadequate – hPSC colonies were too small – High density of hPSC colonies leading to over–crowding – Mycoplasma or other contamination	– Always check hPSC quality before use and perform Mycoplasma testing regularly – Always try to acquire MEFs in bulk and first test their quality with a small lot – Allow hPSC colonies to grow larger and do not use until the optimal day for splitting – Colonies should be sparsely distributed on the 10 cm plates; if colonies are in contact with each other, they tend to detach as larger clusters together with feeders.
22–23	Spheroids stick to each other	– Too many neural spheroids per plate – Spheroids grow quickly once EGF and FGF–2 are added to the media	– Transfer some spheres to a new or bigger low–attachment plate – If already attached, separate them by gently pipetting up and down using a 10 ml pipette when spheroids are young (up to day 25) or two P20 pipette tips when they are older
22–23	Spheroids are too small or disintegrate	– Differentiated hPSC colonies were used – MEFs attached to spheroids after dispase treatment – hPSC colonies were too small – Excessive pipetting – Excessive centrifugation – Mycoplasma or other contamination	– See solutions for step 15 – Be gentle when handling spheroids at all times – Titrate dispase treatment to ensure MEFs remain attached to plate
23, Option A, step II	Unable to assemble hCS and hSS	– hCS or hSS have differentiated too far	– Try to fuse smaller (<3 mm) or early stage spheroids (day 30 to 90). After day 90, the success rate decreases, although older spheroids up to d300 have been successfully fused. – Make sure that spheroids remain in contact in the Eppendorf with minimal disturbance or shaking during the first several days of fusion.
23	Cultures got contaminated	– Inadequate sterile technique – Contamination when transferring between tubes – With very long term cultures, it is important to minimize the time cells are outside the incubator, and to maintain high–level sterile technique	– Improve sterile technique – Wear lab coats – Use 70% ethanol to spray your hands and anything that goes into the safety hood – Autoclave all non–sterile equipment – Change HEPA filters in incubators regularly – Autoclean incubators regularly – Identify sources of contamination in the incubator or cell culture room – Clean water bath in incubators and in the cell culture room on a weekly basis
23	Media turns acidic overnight	– Too many spheroids per plate	– In the initial stages of differentiation, 12–13 ml of media per 10 cm plate is sufficient – As spheroids grow, use 15–18 ml of media per plate – Fewer than 30 spheroids per 10 cm plate are recommended
Box 1, Step 3	Sparse or no viral labeling following transfection	- Viral titer was too low - Insufficient time interval between infection and fluorescent protein expression - Incorrect serotype	- Make sure viral titers are sufficient - Wait at least 1 week before looking for viral gene expression - Ensure that during transfection, spheroids are incubated in a high concentration of virus overnight - Use human DJ serotype for AAV